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Molecular Phylogeny Supports the Validity of *Polypedates impresus* Yang 2008

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Abstract The taxonomy of the Asian tree frogs of the *Polypedates leucomystax* complex, a group of widespread and morphologically similar species, is very controversial. To ascertain the taxonomic status of these species, we investigated the historical relationships among representative samples based on ~ 2 kb of nucleotide sequences from the mitochondrial 12S rRNA, tRNA^{valine}, and 16S rRNA genes. Our phylogeny resolved five well supported lineages (A, B, C, D and E) in the *P. leucomystax* complex. *Polypedates impresus* from Yunnan, China, *Polypedates* cf. *mutus* and *Polypedates* cf. *megacephalus* from Guangxi and Yunnan, China, and Laos constructed Clade C, which is monophyletic. In order to recognize the unique position of this clade, we considered *P. impresus* in Clade C as a valid species. Following our phylogeny, Chinese *Polypedates*, corresponding to the other four clades, should include four species: *P. mutus* (Clade A); *P. braueri* (Clade B); *P. impresus* (Clade C) and *P. megacephalus* (Clade D). *P. leucomystax* (Clade E) is mainly distributed in the Malaysia, Indonesia and Philippines.

Keywords Chinese *Polypedates*, nucleotide sequence, *Polypedates leucomystax* complex, taxonomy

1. Introduction

The ability to recognize species is a fundamental aspect of biological science and has important implications for conservation and ecology. The *Polypedates leucomystax* complex: *P. braueri* (Vogt 1911), *P. megacephalus* (Hallowell, 1861), *P. mutus* (Smith, 1940), *P. impresus* (Yang, 2008), and *P. leucomystax* (Gravenhorst, 1829) (Orlov *et al.*, 2001; Kuraishi *et al.*, 2012), is a group notoriously difficult to identify because of their very similar color pattern and body size (Kuraishi *et al.*, 2012). Brown *et al.* (2010) speculated that the current range of *P. leucomystax* in Southeast Asia was much larger than that in the past recent years, and provided the evidence of recent human-mediated range expansion, using mitochondrial gene sequences (16S rDNA) from 266 samples of geographically widespread tree frogs in Southeast Asia, including 244 of *P. leucomystax* and its close relatives. In the study by Brown *et al.* (2010), the majority of the samples were obtained from the Philippines and Indonesia, which were considered as *P. leucomystax* (Riyanto *et al.*, 2011). Recently, Blair *et al.* (2013) used most of the samples from Vietnam and Laos to investigate the evolutionary history of the *P. leucomystax* complex and tested the hypotheses pertaining to climate, geology, and dispersal patterns.

Historically, Chinese samples were designated as *P. leucomystax* (Liu 1950; Liu and Hu, 1961). Hallowell (1861) described *P. megacephalus* as a new species based on the specimen collected from the type locality in Hong Kong, China. Matsui *et al.* (1986) resurrected *P. megacephalus* from the synonymy of *P. leucomystax*, based on chromosomal, morphological and bioacoustic data, and also suggested that mainland China's populations of these frogs required further taxonomic investigation. Chinese taxonomists have consistently regarded the mainland populations as *P. megacephalus* (Fei, 1999; Fei *et al.*, 2005, 2009, 2010; Zhao and Adler, 1993). Liu *et al.* (1960) considered the samples

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with 'striped type' and with no vocal openings from Hekou, Yunnan, China as *P. mutus*. Inger (1999) also suggested those samples with no vocal openings as *P. mutus* throughout southern China. Thus, there are two species in the genus *Polypedates* in China, including *P. megacephalus* and *P. mutus* (Fei *et al.*, 2009, 2010; Zhao and Adler, 1993). Kuraishi *et al.* (2011) inferred that the populations of *P. megacephalus* in Taiwan and Hong Kong, China differ in DNA sequences, advertisement call and morphological characters. Kuraishi *et al.* (2011) inferred that *P. braueri* (Vogt, 1911) was the valid species in Taiwan, China. Recently, Kuraishi *et al.* (2012) used the nuclear genes [4696 bp sequences fragments of brain-derived neurotrophic factor (BDNF), sodium/calcium exchanger 1 (NCX), POMC, Rag-1, Rhod and Tyr genes] to construct the phylogeny of the genus *Polypedates*, and estimated the history of species diversification and biogeography of the *P. leucomystax* complex combining mitochondrial 12S rRNA, tRNA^{val} and 16S rRNA genes. Based on the molecular phylogeny, Kuraishi *et al.* (2012) supported three species in China: *P. braueri* in northern China, *P. megacephalus* and *P. mutus* in southern China. Yang (2008) described two new species *P. impresus* and *P. spinus*, from Yunnan, southern China. Based on the molecular phylogeny and morphological characters, *P. spinus* should be placed in *Rhacophorus* (Matsui and Panha, 2006; Li *et al.*, 2012). Kuraishi *et al.* (2012) studied and analyzed one juvenile collected from Yunnan and did not confirm it as *P. impresus*. Therefore, the validity of the species needs to be confirmed.

Herein, based on the taxonomically most comprehensive data set, we expand on the phylogenetic relationships of the *P. leucomystax* complex using partial sequences of mitochondrial DNA genes (12S rRNA, 16S rRNA, the complete t-RNA for valine). Based on the molecular phylogeny, we reevaluated the taxonomy the *P. leucomystax* complex with emphasis on Chinese samples and reassessed the validity and taxonomic characters of *P. impresus*.

2. Materials and Methods

2.1 Taxon sampling and data collection Five species, including 128 samples, of the *P. leucomystax* complex were selected as ingroup taxa (Table 1), and five species (*P. colletti*, *P. macrotis*, *P. maculatus*, *P. otilophus*, and *Polypedates* sp. from Malaysia) were chosen as outgroup (Kuraishi *et al.*, 2012). All novel sequences produced in this study are deposited in GenBank with their accession numbers shown in Table 1, and we also obtained some sequences from GenBank (Table 1).

2.2 DNA extraction, amplification and sequencing Genomic DNA was extracted from toeclips, muscle, or liver tissues stored in 95% or 100% ethanol. Tissue samples were digested using proteinase K, and were then treated following a standard 3-step phenol/chloroform extraction procedure (Sambrook *et al.*,, 1989; Hillis *et al.*,, 1996). The mtDNA fragment included 2056 bp from the 12S rRNA and 16S rRNA together with the complete t-RNA for valine. The primers used in this study followed Bossuyt and Milinkovitch (2000), and Wilkinson *et al.* (2002). Double stranded polymerase chain reaction (PCR) amplification for the mitochondrial genes was carried out using the following parameters: initial hot start at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Final extension at 72 °C was conducted for 10 min. Purified PCR products were directly sequenced with an ABI 3730 automated DNA sequencer and sequences were then determined in both directions for each species and submitted for BLAST searching (Altschul *et al.*,, 1997) in GenBank to ensure that the required sequences had been amplified.

2.3 Sequence alignment Alignments were first conducted using program Clustalx 1.81 (Thompson *et al.*,, 1997) with default parameters, and subsequently adjusted by eye. Gaps resulting from the alignment were treated as missing data. Considering that all mtDNA gene sequences are effectively inherited as one locus, the 12S and 16S ribosomal gene fragments and the complete t-RNA for valine were concatenated into a single fragment for the analyses.

2.4 Phylogenetic analyses Possible saturation of substitution types was checked by plotting the number of transitions (Ti) and transversions (Tv) versus TN93 distance using DAMBE (Xia, 2000). The best fitting models of sequence evolution were obtained by ModelTest 3.7 (Posada and Crandall, 1998) for Bayesian inference (BI). The best fitting nucleotide substitution model was selected by using the Akaike Information Criterion (AIC) as implemented in ModelTest 3.7 (Posada and Crandall, 1998). The best-fit models for mtDNA were GTR+I+G. BI was implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and the following settings were applied: number of Markov chain Monte Carlo (MCMC) generations = 50 00 000 and sampling frequency = 100. The first 125 000 sampled trees were discarded as a conservative burn-in. The remaining samples were used to generate a majority rule consensus

Table 1 Samples and sequences used in this study.

Previous name	Present name	Voucher No.	Locality	mtDNA accession No.
Polypedates otilophus	*Polypedates otilophus*	KUHE 42555	Kanowit, Sarawak, Malaysia	AB728190
P. colletti	*P. colletti*	MZB UN	Lampung, Sumatra, Indonesia	AB728189
P. maculatus	*P. maculatus*	KUHE 42336	Rajgir, Bihar, India	AB728188
P. macrotis	*P. macrotis*	KUHE 42556	Kanowit, Sarawak, Malaysia	AB728187
Polypedates sp. Malay	*Polypedates* sp.	KUHE 52552	Temerloh, Pahang, Malaysia	AB728185
Polypedates sp. Malay	*Polypedates* sp.	KUHE 52603	Hut. Lip. Kanching Selangor, Malaysia	AB728177
Polypedates sp. Malay	*Polypedates* sp.	KUHE 53317	Kenaboi, N. Sembilan, Malaysia	AB728178
Polypedates sp. Malay	*Polypedates* sp.	KUHE 52111	Endau Rompin, Johor, Malaysia	AB728184
Polypedates cf. *mutus*	*P. mutus*	CIB 20081090	Wuzhishan, Hainan, China	AB728019
P. megacephalus	*P. mutus*	LSUHC 4220	Wuzhishan, Hainan, China	HM770156
P. cf. *leucomystax*	*P. mutus*	MVZ 236721	Bawangling, Hainan, China	HM770157
P. cf. *leucomystax*	*P. mutus*	MVZ 236715	Bawangling, Hainan, China	HM770155
P. leucomystax	*P. mutus*	FMNH 253029	Vietnam	GQ204751
P. leucomystax	*P. mutus*	FMNH 256451	Laos	GQ204754
P. cf. *mutus*	*P. mutus*	MVZ 226419	Tam Dao, Vinh Phu, Vietnam	AB728023
P. cf. *mutus*	*P. mutus*	ZISP 24603	Kon Plong, Kon Tum, Vietnam	AB728028
P. leucomystax	*P. mutus*	AMNHA 161395	Huong Son, Ha Tinh, Vietnam	DQ283048
P. cf. *mutus*	*P. mutus*	CIB 2851K	Jingxi, Guangxi, China	AB728018
P. cf. *mutus*	*P. mutus*	CIB GX20071159	Jinxiu, Guangxi, China	AB728017
P. cf. *mutus*	*P. mutus*	KUFS 358	Shiwandashang Nature Reserve, Guangxi, China	HM770154
P. cf. *mutus*	*P. mutus*	KUFS 354	Shiwandashang Nature Reserve, Guangxi, China	HM770153
P. aff. *leucomyatax*	*P. mutus*	TZ 98108	Vietnam	AF285223
P. aff. *leucomyatax*	*P. mutus*	TZ 9888	Vietnam	AF285222
P. cf. *leucomystax*	*P. mutus*	MVZ 232163	Vinh Phuc, Vietnam	HM770150
P. cf. *leucomystax*	*P. mutus*	MVZ 226440	Vinh Phuc, Vietnam	HM770152
P. cf. *leucomystax*	*P. mutus*	MVZ 232164	Vinh Phuc, Vietnam	HM770151
P. cf. *leucomystax*	*P. mutus*	MVZ 226451	Vinh Phuc, Vietnam	HM770149
P. mutus	*P. mutus*	IEBRD 690	--	AY880521
P. cf. *mutus*	*P. mutus*	JMG 262	Jing Xin, Guangxi, China	HM770146
P. cf. *leucomystax*	*P. mutus*	MVZ 226439	Vinh Phuc, Vietnam	HM770147
P. braueri	*P. braueri*	KUHE 37040	Longchuan, Yunnan, China	AB728016
P. braueri	*P. braueri*	SCUM 060602L	Tengchong, Yunnan, China	EU215553
P. braueri	*P. braueri*	CIBGX 200807039	Jinxiu, Guangxi, China	AB728007
P. braueri	*P. braueri*	CIB UN	Tianlin, Guangxi, China	AB728010
P. cf. *megacephalus*	*P. braueri*	JMG 313	Jing Xin, Guangxi, China	HM770145
P. braueri	*P. braueri*	CIB 2855K	Jingxi, Guangxi, China	AB728008
P. megacephalus	*P. braueri*	LJT 010	Puer, Yunnan, China	KF053205*
P. megacephalus	*P. braueri*	LJT 011	Puer, Yunnan, China	KF053216*
P. megacephalus	*P. braueri*	LJT 012	Puer, Yunnan , China	KF053204*
P. megacephalus	*P. braueri*	LC 0805096	Fenshuiling, Lüchun, Yunnan, China	KF053210*
P. megacephalus	*P. braueri*	LC 0805095	Fenshuiling, Lüchun, Yunnan, China	KF053211*
P. megacephalus	*P. braueri*	LJT 201105119	Xuanhan, Sichuan, China	KF053213*
P. braueri	*P. braueri*	KUHE 43184	Hejiang, Sichuan, China	AB728011
P. megacephalus	*P. braueri*	LJT 20110411	Yibin, Sichuan, China	KF053214*
P. megacephalus	*P. braueri*	LJT 201105120	Xuanhan, Sichuan, China	KF053212*
P. megacephalus	*P. braueri*	LJT 73	Ya'an, Sichuan, China	KF053220*
P. braueri	*P. braueri*	KUHE 12971	Riyue Tan, Nantou, China	AB564278
P. megacephalus	*P. braueri*	--	--	AF026350
P. megacephalus	*P. braueri*	--	--	AF458141
P. braueri	*P. braueri*	KUHE 32842	Taipei, China	AB564281
P. braueri	*P. braueri*	CIBGX200905083	Hezhou, Guangxi, China	AB728005
P. braueri	*P. braueri*	KUHE 33625	Nanning, Guangxi, China	AB728009
P. braueri	*P. braueri*	KUHE 33626	Ziyuan, Guangxi, China	AB728003
P. braueri	*P. braueri*	KUHE 32931	Huangshan, Anhui, China	AB564282
P. braueri	*P. braueri*	CIBJX 200806182	Zixi, Jiangxi, China	AB728001
P. braueri	*P. braueri*	SCUM 050508C	Mt. Daiyun, Fujian, China	EU215552
P. megacephalus	*P. braueri*	LJT 83DD	Chebaling, Guangdong, China	KF053218*

(Continued Table 1)

Previous name	Present name	Voucher No.	Locality	mtDNA accession No.
P. megacephalus	*P. braueri*	LJT 84DD	Chebaling, Guangdong, China	KF053217*
P. megacephalus	*P. braueri*	LJT 85DD	Chebaling, Guangdong, China	KF053215*
P. braueri	*P. braueri*	CIBZJ 200806267	Jiande, Zhejiang, China	AB727999
P. braueri	*P. braueri*	CIBZJ 200806091	Tiantai, Zhejiang, China	AB728000
P. impresus	*P. impresus*	LC 0805072	Lüchun, Yunnan, China	KF053208*
P. cf. *mutus*	*P. impresus*	SCUM 37940C	Xishuangbanna, Yunnan, China	EU215551
P. cf. *mutus*	*P. impresus*	KUHE 32448	Ban Saleui, Houa Phan, Laos	AB728169
P. cf. *mutus*	*P. impresus*	KUHE 40698	Ban Nam Tian, Xieng Khouang, Laos	AB728174
P. cf. *mutus*	*P. impresus*	JMG 308	Jing Xin, Guangxi, China	HM770158
P. cf. *mutus*	*P. impresus*	JMG 310	Jing Xin, Guangxi, China	HM770159
P. cf. *megacephalus*	*P. impresus*	JMG 301	Jing Xin, Guangxi, China	HM770160
P. cf. *mutus*	*P. impresus*	JMG 302	Jing Xin, Guangxi, China	HM770161
P. cf. *megacephalus*	*P. impresus*	JMG 287	Jing Xin, Guangxi, China	HM770162
P. impresus	*P. impresus*	LC 0805099	Lüchun,Yunnan, China	KF053206*
P. impresus	*P. impresus*	LC 0805100	Lüchun,Yunnan, China	KF053207*
P. impresus	*P. impresus*	LC 0805113	Lüchun, Yunnan, China	KF053209*
P. leucomystax	*P. megacephalus*	MVZ 226407	Vinh Phuc, Vietnam	HM770166
P. megacephalus	*P. megacephalus*	KUHE 38470	Tam Dao, Vinh Phu, Vietnam	AB728036
P. megacephalus	*P. megacephalus*	KUHE 38477	Hanoi, Vinh Phu, Vietnam	AB728045
P. leucomystax	*P. megacephalus*	MVZ 226396	Vinh Phuc, Vietnam	HM770163
P. leucomystax	*P. megacephalus*	MVZ 223834	Vinh Phuc, Vietnam	HM770164
P. leucomystax	*P. megacephalus*	MVZ 232165	Vinh Phuc, Vietnam	HM770165
P. leucomystax	*P. megacephalus*	KUHE 32929	Hong Kong, China	AB564283
P. megacephalus	*P. megacephalus*	SCUM 0607116L	Huidong, Guangdong, China	EU215550
P. megacephalus	*P. megacephalus*	LJT 1SDD	Nonggang, Guangxi, China	KF053219*
P. megacephalus	*P. megacephalus*	CIB 07033	Diaoluoshan, Hainan, China	AB728034
P. megacephalus	*P. megacephalus*	CIBGX 200807047	Jinxiu, Guangxi, China	AB728032
P. cf. *megacephalus*	*P. megacephalus*	LSUHC 4155	Bawangling, Hainan, China	HM770172
P. cf. *leucomystax*	*P. megacephalus*	MVZ 236719	Bawangling, Hainan, China	HM770169
P. cf. *leucomystax*	*P. megacephalus*	MVZ 236720	Bawangling, Hainan, China	HM770170
P. cf. *leucomystax*	*P. megacephalus*	MVZ 236716	Bawangling, Hainan, China	HM770171
P. megacephalus	*P. megacephalus*	ZISP 39244	Kon Plong, Kon Tum, Vietnam	AB728067
P. megacephalus	*P. megacephalus*	KUHE 40543	Hue, Thua Thien-Hue, Vietnam	AB728051
P. cf. *mutus*	*P. megacephalus*	KUFS 360	Shiwandashang Nature Reserve, Guangxi, China	HM770168
P. cf. *megacephalus*	*P. megacephalus*	KUFS 026	Nanning, Guangxi, China	HM770167
P. leucomystax	*P. megacephalus*	FMNH 253086	Vietnam	GQ204752
P. megacephalus	*P. megacephalus*	KUHE 40521	A Luoi, Thua Thien-Hue, Vietnam	AB728061
P. megacephalus	*P. megacephalus*	KUHE 20219	Ko Chang, Trat, Thailand	AB728091
P. leucomystax	*P. megacephalus*	FMNH 255296	Laos	GQ204753
P. megacephalus	*P. megacephalus*	KUHE 22266	Pupha Namtip, Roi Et, Thailand	AB728080
P. megacephalus	*P. megacephalus*	KUHE 40650	Vientiane, Laos	AB728068
P. megacephalus	*P. megacephalus*	KUHE 22196	Nong Khai, Thailand	AB728077
P. megacephalus	*P. megacephalus*	KUHE 20033	Kaeng Krachang, Phetchaburi, Thailand	AB728086
P. megacephalus	*P. megacephalus*	KUHE 34155	Khao Khitchakut, Chanthaburi, Thailand	AB728087
P. megacephalus	*P. megacephalus*	KUHE 20597	Mae Yom, Phrae, Thailand	AB728075
P. megacephalus	*P. megacephalus*	KUHE 19904	Tha Ton, Chiang Mai, Thailand	AB728073
P. megacephalus	*P. megacephalus*	KUHE 19196	Doi Pui, Chiang Mai, Thailand	AB728074
P. megacephalus	*P. megacephalus*	KUHE 20373	Khao Sa Bap, Chanthaburi, Thailand	AB728088
P. megacephalus	*P. megacephalus*	KUHE 23953	Sakaerat, Nakorn Ratchasima, Thailand	AB728081
P. megacephalus	*P. megacephalus*	KUHE 19470	Songkhla Buri, Kanchanaburi, Thailand	AB728082
P. leucomystax	*P. leucomystax*	KUHE 33880	Chatthin, Myanmar	AB728167
P. leucomystax	*P. leucomystax*	MZB UN	Temanggung, Java, Indonesia	AB728135
P. leucomystax	*P. leucomystax*	JAM 3211	Lombok Island, Indonesia	HM770313
P. leucomystax	*P. leucomystax*	MZB Amp 12864	Banyuwangi, Java, Indonesia	AB728137
P. leucomystax	*P. leucomystax*	--	Jave, Indonesia	AF026351
P. leucomystax	*P. leucomystax*	MZB UN	Yogyakarta, Java, Indonesia	AB728136

(Continued Table 1)

Previous name	Present name	Voucher No.	Locality	mtDNA accession No.
P. leucomystax	*P. leucomystax*	KUHE 34396	Iloilo, Panay, Philippines	AB728164
P. leucomystax	*P. leucomystax*	RMB 3343	Camarines Sur, Philippines	HM770350
P. leucomystax	*P. leucomystax*	--	Java, Indonesia	AF458140
P. leucomystax	*P. leucomystax*	KU 302446	Guimaras, Philippines	HM770340
P. leucomystax	*P. leucomystax*	PT 11	Lubuk Sikaping, Sumatra, Indonesia	AB728130
P. leucomystax	*P. leucomystax*	RMB 2088	Jawa Barat, Indonesia	HM770262
P. leucomystax	*P. leucomystax*	MZB UN	Depok, Java, Indonesia	AB564285
P. leucomystax	*P. leucomystax*	ZRC 15269	Java, Indonesia	AY141849
P. leucomystax	*P. leucomystax*	JAM 4453	Kendari, Sulawesi, Indonesia	AB728159
P. leucomystax	*P. leucomystax*	MZB Amp 8026	Banggai, Sulawesi, Indonesia	AB728155
P. leucomystax	*P. leucomystax*	TNHC 58967	Sulawesi Tengah, Indonesia	HM770227
P. leucomystax	*P. leucomystax*	RMB 1625	Sulawesi Tengah, Indonesia	HM770223
P. leucomystax	*P. leucomystax*	MVZ 255017	Sulawesi Tengah, Indonesia	HM770228
P. leucomystax	*P. leucomystax*	TNHC 59794	Sulawesi Utara, Indonesia	HM770247
P. leucomystax	*P. leucomystax*	MZB Amp 8024	Bolaang Mongondow, Sulawesi, Indonesia	AB728154
P. leucomystax	*P. leucomystax*	MVZ 255112	Sulawesi Selatan, Indonesia	HM770190
P. leucomystax	*P. leucomystax*	KUHE 20790	Toraja, Sulawesi, Indonesia	AB728156
P. leucomystax	*P. leucomystax*	MZB Amp 12444	Polewali, Sulawesi, Indonesia	AB728158
P. leucomystax	*P. leucomystax*	MZB Amp 12884	Soppeng, Sulawesi, Indonesia	AB728160
P. leucomystax	*P. leucomystax*	MZB UN	Benteng, Selayar, Indonesia	AB728162
P. leucomystax	*P. leucomystax*	MVZ 255117	Sulawesi Selatan, Indonesia	HM770182
P. leucomystax	*P. leucomystax*	KUHE 23555	Brostagi, Sumatra, Indonesia	AB728128
P. leucomystax	*P. leucomystax*	--	Jave, Indonesia	AF215343
P. leucomystax	*P. leucomystax*	KUHE 19546	Ko Samui, Surat Thani, Thailand	AB728096
P. leucomystax	*P. leucomystax*	KUHE 2386	Ranong, Thailand	AB728092
P. leucomystax	*P. leucomystax*	KUHE 53147	Kapit, Sarawak, Malaysia	AB728148
P. leucomystax	*P. leucomystax*	KUHE 39074	Langkawi, Kedah, Malaysia	AB728102
P. leucomystax	*P. leucomystax*	KUHE 12042	Matang, Sarawak, Malaysia	AB728152
P. leucomystax	*P. leucomystax*	KUHE:52162	Endau Rompin, Johor, Malaysia	AB728123
P. leucomystax	*P. leucomystax*	KUHE 53289	Ledang, Johor, Malaysia	AB728118
P. leucomystax	*P. leucomystax*	KUHE 53248	Ampang, Selangor, Malaysia	AB728109
P. leucomystax	*P. leucomystax*	KUHE 53344	Kenaboi, Negeri Sembilan, Malaysia	AB728115
P. leucomystax	*P. leucomystax*	KUHE 15157	Fraser's Hill, Pahang, Malaysia	AB728108
P. leucomystax	*P. leucomystax*	KUHE 15774	Kuala Lumpur, Malaysia	AB728116
P. leucomystax	*P. leucomystax*	KUHE 52576	Kanching Selangor, Malaysia	AB728114
P. leucomystax	*P. leucomystax*	KUHE 15036	Gombak, Selangor, Malaysia	AB728112
P. leucomystax	*P. leucomystax*	KUHE 19342	Nakon Si Tammarat, Nakon Si Tammarat, Thailand	AB728097
P. leucomystax	*P. leucomystax*	KUHE 23395	Khao Chong, Trang, Thailand	AB728098
P. leucomystax	*P. leucomystax*	KUHE 19630	Khlong Saeng, Surat Thani, Thailand	AB728094
P. leucomystax	*P. leucomystax*	KUHE 52375	Lata Tembakoh, Terengganu, Malaysia	AB728110
P. leucomystax	*P. leucomystax*	KUHE 23173	Hala-Bala, Narathiwat, Thailand	AB728101
P. leucomystax	*P. leucomystax*	KUHE 15316	Jerai, Kedah, Malaysia	AB728103
P. leucomystax	*P. leucomystax*	KUHE 15236	Pinang, P. Pinang, Malaysia	AB728104
P. leucomystax	*P. leucomystax*	FMNH 244921	Sabah, Malaysia	HM770295
P. leucomystax	*P. leucomystax*	BORN 12420	Kota Kinabalu, Sabah, Malaysia	AB728138
P. leucomystax	*P. leucomystax*	BORN 23373	Keningau, Sabah, Malaysia	AB728143
P. leucomystax	*P. leucomystax*	BORN 22430	Tampaluri, Sabah, Malaysia	AB728144
P. leucomystax	*P. leucomystax*	FMNH 244922	Sabah, Malaysia	HM770266
P. leucomystax	*P. leucomystax*	FMNH 231040	Sabah, Malaysia	HM770281

[*] Sequences new to this study. AMNH: American Museum of Natural History, New York, USA; BORN: BORNEENSI collection, the University Malaysia Sabah; CIB: Chengdu Institute of Biology, the Chinese Academy of Sciences, Chengdu, China; FMNH: Field Museum of Natural History, Chicago, USA; KUHE: Kyoto University, Graduate School of Human and Environmental Studies, Japan; MVZ: Museum of Vertebrate Zoology, University of California Berkeley, USA; MZB: the United States National Museum and the Zoological Institute, Saint Petersburg, USA; SCUM: Sichuan University Museum, Sichuan, China; ZRC: Zoological Reference Collection, Raffles Museum of Biodiversity Research, Singapore. LC = Lüchun, Yunnan, China; LJT = field numbers given by Jiatang LI. "--" = unknown data.

tree. The frequency of resolving a node was termed a Bayesian posterior probability (BPP). All MCMC runs were repeated twice to confirm consistent approximation of the posterior parameter distributions.

3. Results

3.1 Sequence variation The aligned mtDNA gene fragments consisted of 2410 nucleotide positions, corresponding to the mitochondrial DNA genes (12S rRNA, 16S rRNA, the complete t-RNA for valine). Plots of transitions and transversions showed a linear relationship, thus giving no indication of saturation effects. Consequently, all substitutions and codon positions in these genes were used for phylogenetic inference.

3.2 Phylogenetic analysis For BI, the likelihood value of the 50% majority consensus tree was lnL = -13546.96. The standard deviation of split frequencies among the four BI runs was 0.00121. The following relationships were indicated by BI methods of analyses as being well supported and reliable. The *P. leucomystax* complex was a strongly supported lineage with respect to the outgroup (BPP = 1.00). Clade A included the samples from Vietnam, and Guangxi and Hainan, China with strong support (BPP = 1.00), which was basal to the remaining samples in the *P. leucomystax* complex. Clade B, containing the samples from Anhui, Fujian, Guangxi, Jiangxi, Sichuan, Taiwan, Zhejiang, and Yunnan, China (BPP = 1.00) was sister to the group of clades C, D and E. The samples from Guangxi and Yunnan of China, and Laos formed Clade C (BPP = 1.00). The samples from Guangdong, Hainan, Guangxi and Hong Kong of China, Vietnam, Thailand and Laos were included in Clade D with strong support (BPP = 1.00). Clade E included the samples from Myanmar, Indonesia, Philippines and Thailand (BPP = 1.00). Clade D was sister to Clade E with strong support (BPP = 1.00), and together formed a sister group to Clade C (BPP = 1.00).

4. Discussion

4.1 Validity of *Polypedates impresus* Yang 2008 Traditional taxonomic classifications based largely on comparisons of overall morphological similarity (Dubois, 1992; Fei *et al.*, 2005) are now unstable because of extensive homoplasy in both larvae and adults (Bossuyt and Milinkovitch, 2000; Stuart, 2008). Morphological variations may result from phenotypic plasticity and adaptation to local environments, which may mislead our understanding of evolutionary relationships (Herrmann *et al.*, 2004). In this regard, molecular data, including phylogenetic and genealogical reconstructions, could provide much needed additional information.

Six specimens of *P. impresus* were collected from Lüchun, Yunnan, southern China during fieldwork in 2008. Based on the original description by Yang (2008), we considered four of our samples to be *P. impresus* due to the following characters (Figure 1): the top of head obviously concave, upper lip margin white, dorsal body light brown, and with no zebra-like stripes. Another two samples were morphologically different and were not considered as *P. impresus*.

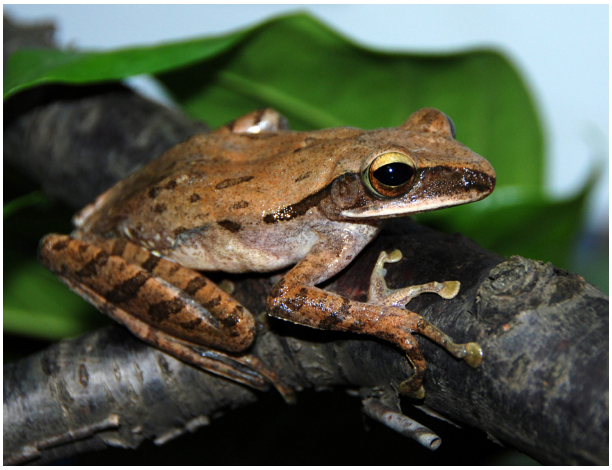


Figure 1 *Polypedates impresus* collected from Lüchun, Yunnan, southern China during fieldwork in 2008.

Adding the sequences of *P. impresus*, our phylogeny recovered that four samples of *P. impresus*, *P.* cf. *mutus* and *P.* cf. *megacephalus* from Yunnan and Guangxi, China, and Laos formed a clade, that is, monophyletic Clade C. Consistent with the study by Kuraishi *et al.* (2012), Clade C is unique. In order to recognize the unique position of this clade, we provisionally consider it to be a valid species and considered Clade C as *P. impresus*.

Smith (1940) described the new species *P. mutus* with its type locality in N'Chang Yang, northern Myanmar and the samples have several dark stripes on their dorsum and lack vocal openings. Lu *et al.* (2010) suggested that a mixed color pattern of 'X' shaped dark stripes on the dorsum of the trunk is not correlated with geographic distribution and could not be used to distinguish it as a different species. Li *et al.* (2012) also indicated that color patterns should not be considered as a taxonomic character. We cannot resolve the clade *P.* cf. *mutus* as true *P. mutus*, and more data from the type locality are needed. Kuraishi *et al.* (2012) included one sample from

Longchuan, Yunnan and the sample differs from the other samples with the relatively high divergence. Because the sample is a juvenile and lacks taxonomically important features, Kuraishi *et al.* (2012) could not discuss the exact status of this sample. Our samples from Tengchong and Puer, Yunnan, the sample from Longchuan, Yunnan by Kuraishi *et al.* (2012) and the samples from other areas of China, such as Guangdong, Sichuan and Guangxi, were nested within Clade B, and the sample from the study by Kuraishi *et al.* (2012) was still highly divergent from others. In this study, we provisionally considered all samples of this clade as *P. braueri*. As currently understood, there are two species in Yunnan, China, *P. braueri* and *P. impresus*.

4.2 *Polypedates leucomystax* complex in China In this study, we combined the previously published DNA data, including those by Brown *et al.* (2010), Li *et al.* (2008, 2009), and Kuraishi *et al.* (2012). Five clades (A, B, C, D and E) were recovered in our phylogeny. Clade C was considered as *P. impresus*. Clades B, D and E included the samples of *P. braueri* from the type locality in Taiwan, China, *P. megacephalus* from the type locality in Hong Kong, China, and *P. leucomystax* from the type locality in Java. We considered clades B, D and E as *P. braueri*, *P. megacephalus* and *P. leucomystax*, respectively.

Historically, Liu *et al.* (1960) considered the samples with the 'striped type' and with no vocal openings from Hekou, Yunnan, China as *P. mutus*. Inger *et al.* (1999) also suggested those samples with no vocal openings should be *P. mutus* distributed in southern China. Hallowell (1861) described *P. megacephalus* as a new species based on the specimen collected from its type locality in Hong Kong, China. Matsui *et al.* (1986) resurrected *P. megacephalus* from the synonymy of *P. leucomystax* based on chromosomal, morphological, and bioacoustic data, and suggested that mainland China's populations of these frogs required further taxonomic investigation. In geography, mainland China's populations of *P. megacephalus* were nearer to the type locality in Hong Kong, China than the type locality "Java" for *P. leucomystax* in Indonesia. Thus, Chinese taxonomists have consistently regarded mainland China's populations as *P. megacephalus* (Fei, 1999; Fei *et al.*, 2005, 2009, 2010; Zhao and Adler, 1993). There are two species in the genus *Polypedates* in China, that is, *P. megacephalus* and *P. mutus* (Fei *et al.*, 2009, 2010; Zhao and Adler, 1993). Kuraishi *et al.* (2011) inferred that the populations of *P. megacephalus* in Taiwan and Hong Kong, China differ in DNA sequences, advertisement call and morphological characters. Thus, Kuraishi *et al.* (2011) recovered the name *P. braueri* (Vogt, 1911) for the type locality, Taiwan, China. Recently, Kuraishi *et al.* (2012) resolved the taxonomy of the *P. leucomystax* complex and considered there are three species in China.

Following our phylogeny, Chinese *Polypedates* samples, corresponding to four clades, should include four species: *Polypedates* cf. *mutus* from Guangxi and Hainan, China (Clade A), *P. braueri* from Anhui, Fujian, Guangxi, Jiangxi, Sichuan, Taiwan, Zhejiang, and Yunnan, China (Clade B), *P. impresus* from Guangxi and Yunnan, China (Clade C), and *P. megacephalus* from Guangdong, Hainan, Guangxi and Hong Kong, China (Clade D). *P. leucomystax* (Clade E) is mainly distributed in the Philippines and Sulawesi, Indonesia.

Several incidences of sympatric lineages occur in the *P. leucomystax* complex (Blair *et al.*, 2013; Kuraishi *et al.*, 2012), such as two species *P. impresus* and *P. braueri* in Lüchun, Yunnan, China in our study (Figure 2).

4.3 Description of *Polypedates impresus* Yang 2008 The text below was translated from the Chinese literature (Yang and Rao, 2008) (Table 2).

Holotype: No. 01092; an adult male; Nuozhadu, Simao (= Puer at present), Yunnan, China; altitude 850 m; collected on 5 June 2001 by Datong YANG.

Allotype: No. 01070; an adult female; collected from the same locality as the holotype.

Diagnosis: The top of head obviously concave; upper lip margin white; dorsal body light brown, and with no

Table 2 Measurements of *Polypedates impresus* Yang sp. nov. (in mm) (Puer: Nuozhadu).

Number	Locality	Sex	SVL	HL	HW	SL	IOD	FLL	HLT	FTL	TIL	FL
01091	Puer	Male	49.5	18	16	8	6	22	14	81	25	20.5
01092	Puer	Female	65	23.5	23.5	11	7	32	20.5	108	36.5	30
01070	Puer	Female	75	26	23.5	12	8.5	33.5	20.5	105	38	31.5

SVL: Snout-vent length (from tip of snout to vent); HL: Head length (from tip of snout to the posterial border of the angle of the jaw, and not measured parallel with the median line); HW: Head width (at angle of jaw); SL: Snout length (from anterior border of eye to tip of snout); IOD: Interorbital distance (minimum distance between upper eyelids); FLL: Forelimb length (from elbow to tip of finger III); HLT: Hand length (from base of outer palmer tubercle to tip of finger III); FTL: Length of foot and tarsus; TIL: Tibia length (from knee to foot); FL: Foot length (from proximal end of inner metatarsal tubercle to tip of toe IV).

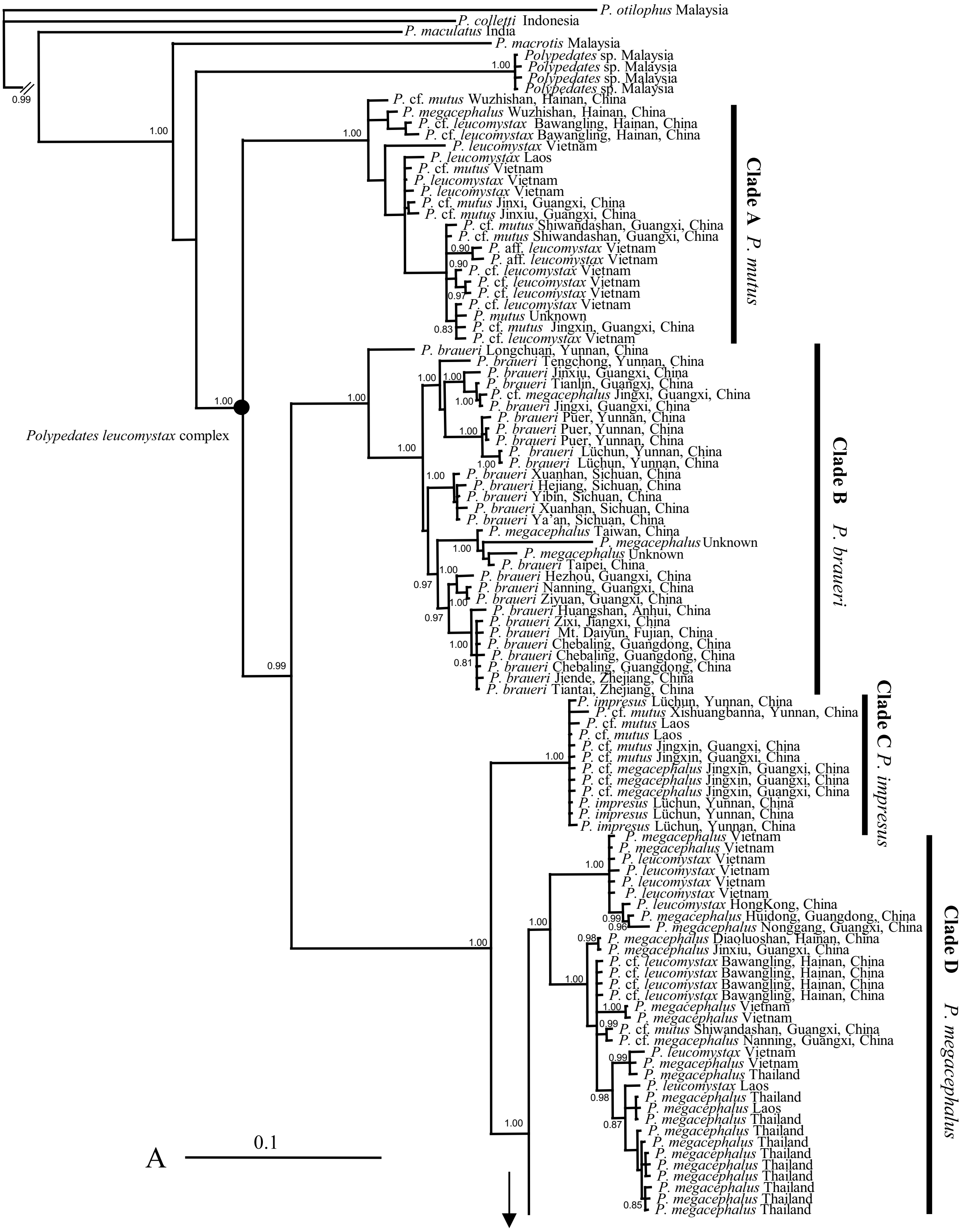
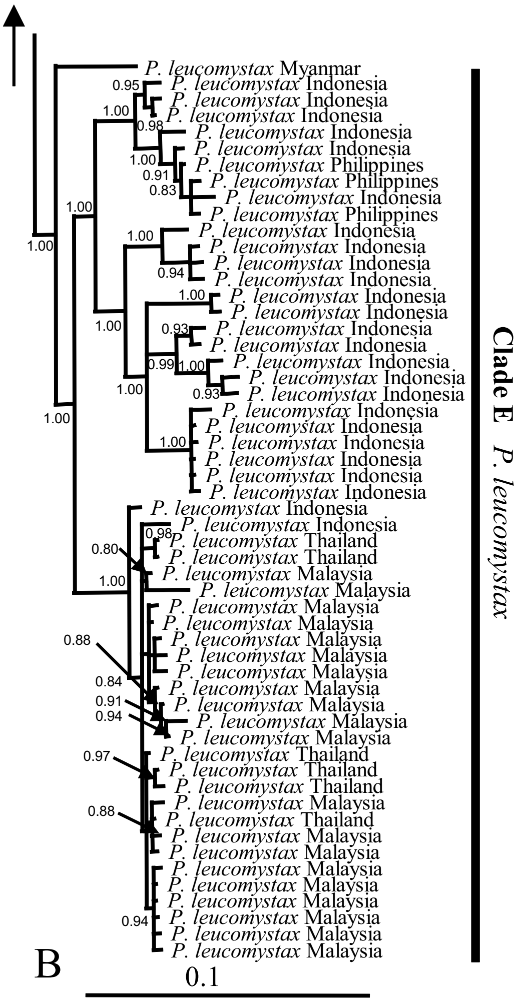

P. otilophus Malaysia
P. colletti Indonesia
P. maculatus India
P. macrotis Malaysia
Polypedates sp. Malaysia
Polypedates sp. Malaysia
Polypedates sp. Malaysia
Polypedates sp. Malaysia
P. cf. mutus Wuzhishan, Hainan, China
P. megacephalus Wuzhishan, Hainan, China
P. cf. leucomystax Bawangling, Hainan, China
P. cf. leucomystax Bawangling, Hainan, China
P. leucomystax Vietnam
P. leucomystax Laos
P. cf. mutus Vietnam
P. leucomystax Vietnam
P. leucomystax Vietnam
P. cf. mutus Jinxi, Guangxi, China
P. cf. mutus Jinxiu, Guangxi, China
P. cf. mutus Shiwandashan, Guangxi, China
P. cf. mutus Shiwandashan, Guangxi, China
P. aff. leucomystax Vietnam
P. aff. leucomystax Vietnam
P. cf. leucomystax Vietnam
P. cf. leucomystax Vietnam
P. cf. leucomystax Vietnam
P. cf. leucomystax Vietnam
P. mutus Unknown
P. cf. mutus Jingxin, Guangxi, China
P. cf. leucomystax Vietnam
Clade A P. mutus
P. braueri Longchuan, Yunnan, China
P. braueri Tengchong, Yunnan, China
P. braueri Jinxiu, Guangxi, China
P. braueri Tianlin, Guangxi, China
P. cf. megacephalus Jingxi, Guangxi, China
P. braueri Jingxi, Guangxi, China
P. braueri Puer, Yunnan, China
P. braueri Puer, Yunnan, China
P. braueri Puer, Yunnan, China
P. braueri Lüchun, Yunnan, China
P. braueri Lüchun, Yunnan, China
P. braueri Xuanhan, Sichuan, China
P. braueri Hejiang, Sichuan, China
P. braueri Yibin, Sichuan, China
P. braueri Xuanhan, Sichuan, China
P. braueri Ya'an, Sichuan, China
P. megacephalus Taiwan, China
P. megacephalus Unknown
P. megacephalus Unknown
P. braueri Taipei, China
P. braueri Hezhou, Guangxi, China
P. braueri Nanning, Guangxi, China
P. braueri Ziyuan, Guangxi, China
P. braueri Huangshan, Anhui, China
P. braueri Zixi, Jiangxi, China
P. braueri Mt. Daiyun, Fujian, China
P. braueri Chebaling, Guangdong, China
P. braueri Chebaling, Guangdong, China
P. braueri Chebaling, Guangdong, China
P. braueri Jiende, Zhejiang, China
P. braueri Tiantai, Zhejiang, China
Clade B P. braueri
P. impresus Lüchun, Yunnan, China
P. cf. mutus Xishuangbanna, Yunnan, China
P. cf. mutus Laos
P. cf. mutus Laos
P. cf. mutus Jingxin, Guangxi, China
P. cf. mutus Jingxin, Guangxi, China
P. cf. megacephalus Jingxin, Guangxi, China
P. cf. megacephalus Jingxin, Guangxi, China
P. cf. megacephalus Jingxin, Guangxi, China
P. impresus Lüchun, Yunnan, China
P. impresus Lüchun, Yunnan, China
P. impresus Lüchun, Yunnan, China
Clade C P. impresus
P. megacephalus Vietnam
P. megacephalus Vietnam
P. leucomystax Vietnam
P. leucomystax Vietnam
P. leucomystax Vietnam
P. leucomystax Vietnam
P. leucomystax HongKong, China
P. megacephalus Huidong, Guangdong, China
P. megacephalus Nonggang, Guangxi, China
P. megacephalus Diaoluoshan, Hainan, China
P. megacephalus Jinxiu, Guangxi, China
P. cf. leucomystax Bawangling, Hainan, China
P. cf. leucomystax Bawangling, Hainan, China
P. cf. leucomystax Bawangling, Hainan, China
P. cf. leucomystax Bawangling, Hainan, China
P. megacephalus Vietnam
P. megacephalus Vietnam
P. cf. mutus Shiwandashan, Guangxi, China
P. cf. megacephalus Nanning, Guangxi, China
P. leucomystax Vietnam
P. megacephalus Vietnam
P. megacephalus Thailand
P. leucomystax Laos
P. megacephalus Thailand
P. megacephalus Laos
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
P. megacephalus Thailand
Clade D P. megacephalus
Polypedates leucomystax complex
0.99
1.00
0.90
0.97
0.83
0.81
0.96
0.98
0.87
0.85
A
0.1

Figure 2 Bayesian inference tree derived from the part of 12S and 16S ribosomal genes together the complete t-RNA for valine. Numbers above the lines or besides the nodes are Bayesian posterior probabilities (≥ 90% retained). "--" represents Bayesian posterior probabilities lower than 90%.

(Continued Figure 2)


P. leucomystax Myanmar
0.95 P. leucomystax Indonesia
P. leucomystax Indonesia
1.00 P. leucomystax Indonesia
0.98 P. leucomystax Indonesia
P. leucomystax Indonesia
1.00 P. leucomystax Philippines
0.91 P. leucomystax Philippines
0.83 P. leucomystax Indonesia
1.00 P. leucomystax Philippines
1.00 P. leucomystax Indonesia
1.00 P. leucomystax Indonesia
0.94 P. leucomystax Indonesia
P. leucomystax Indonesia
1.00 P. leucomystax Indonesia
P. leucomystax Indonesia
1.00 0.93 P. leucomystax Indonesia
P. leucomystax Indonesia
0.99 1.00 P. leucomystax Indonesia
P. leucomystax Indonesia
1.00 0.93 P. leucomystax Indonesia
1.00 P. leucomystax Indonesia
P. leucomystax Indonesia
1.00 P. leucomystax Indonesia
P. leucomystax Indonesia
P. leucomystax Indonesia
P. leucomystax Indonesia
P. leucomystax Indonesia
P. leucomystax Indonesia
0.98 P. leucomystax Thailand
0.80 P. leucomystax Thailand
1.00 P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
0.88 P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
0.84 P. leucomystax Malaysia
0.91 P. leucomystax Malaysia
0.94 P. leucomystax Malaysia
P. leucomystax Malaysia
0.97 P. leucomystax Thailand
P. leucomystax Thailand
P. leucomystax Thailand
P. leucomystax Malaysia
0.88 P. leucomystax Thailand
P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
0.94 P. leucomystax Malaysia
P. leucomystax Malaysia
P. leucomystax Malaysia
Clade E P. leucomystax
B
0.1

zebra-like stripes.

Description: The body moderate in size, with snout-vent length 65 mm and 75 mm in male and female; dorsal body light brown; upper lip margin white; tempus with one brown stripe; forearms with two stripes; hindlimbs having broad stripes, which are darker in color than body; lateral body with no stripe; dim and small spots arranged on the rear of thigh; skin of dorsal body smooth, with no tubercles, no dorsolateral folds; temporal fold found almost flat and straight on the upper tympanum.

Head nearly equal in length and width; head, dorsal and ventral body flat; snout protruding sharply, slightly over lower jaw; length of snout > diameter of eye > width of upper eyelid; canthus rostralis easily seen; naris close to the tip of snout; tympanum rounded, smaller than the width of upper eyelid; temporal fold flat and straight; maxillary teeth strong, vomerine teeth long, arranged at the posterior edges of the choane, not meeting; tongue large, deeply notched posteriorly.

Arms and legs long and thin, forearms relatively strong; fingers long and thin, with relative lengths of fingers III > IV > II > I, finger II slightly longer than finger I; finger tip with an expanded disk and horizontal groove; subarticular tubercles small and round, supernumerary tubercles present; three palmar tubercles present, and fingers III and IV with obvious palmar tubercles; hind legs long and thin, twice longer than body length; tibia nearly half as long as body; foot shorter than tibia; toe tip with an expanded disk; toes half webbed, having internal metatarsal tubercles, but no outer metatarsal tubercles; outer metatarsus nearly 1/2 webbed.

Habitat: Growing among thick grasses on small hills along the Lancang River.

Distribution: Currently only found in Nuozhadu, Simao, Yunnan, China.

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